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Perinatal exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin suppresses contextual fear conditioning-accompanied activation of cyclic AMP response element-binding protein in the hippocampal CA1 region of male rats

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Abstract

We investigated the effect of in utero and lactational exposures to dioxin on adult offspring with contextual fear conditioning, a sex- and hippocampus-dependent learning paradigm; and we measured the conditioning-accompanied activation of cyclic AMP response element-binding protein (CREB) in the hippocampal CA1 region. Pregnant rats were treated with a low dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on gestation day 15. TCDD treatment decreased freezing time in conditioning tests of adult male offspring but not of female offspring. A similar, male-specific decrease was observed in the percentage of phosphorylated CREB-immunoreactive neurons in the CA1 region following conditioning in TCDD-treated rats. These results suggest that perinatal TCDD exposure impairs hippocampus-dependent learning in male offspring by suppressing CREB activation.

Keywords: Dioxin; Contextual fear conditioning; Cyclic AMP response element-binding protein (CREB); Sex difference; Hippocampus

Dioxins are ubiquitous environmental contaminants that are carcinogenic, immunotoxic, and toxic for the reproductive system and development [3,21]. Dioxin exposure during embryonic and fetal periods affects the development of offspring even when the exposure level is too low to induce toxicity in the mother [14]. Because the developing central nervous system is vulnerable to teratogenic insults throughout embryonic and fetal periods [2], it is of particular concern that exposure to relatively low levels of dioxins during development leads to alterations in neural functions, in particular learning and memory, of adult brain. Furthermore, because the period from late gestation to immediately after birth in rats corresponds to the critical period for brain sexual differentiation [7], perinatal administration of dioxins and coplanar polychlorinated biphenyls (PCBs) influences sex differences or causes sex-dependent alterations in learning behavior at the adult [24]. Perinatal administration of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic isomer among a group of dioxin compounds, to pregnant rats alters the patterns of schedule-controlled operant performances and cortical lateralization differentially in female and male offspring [8,26]. Higher prenatal dioxin levels are associated with more feminized play in boys and girls as assessed by the feminine scale [22,23]. However, little is known about the cellular and molecular basis for the perinatal dioxin effect on learning ability and the sex difference of the effect.

Cyclic AMP response element-binding protein (CREB) is a transcription factor implicated in learning and memory. Long-term memory is disturbed in species ranging from *Aplysia* to mice when CREB production is blocked [4,5,25]. Learning and long-term potentiation of synaptic transmission are accompanied by spatiotemporal changes in CREB activation in the hippocampus [10,19,20], a crucial neural structure involved in the acquisition and consolidation of many forms of memory. Recently, we have shown that CREB in the CA1 region of the hippocampus is activated in a male-specific manner by contextual fear conditioning [13], a hippocampus-dependent learning

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paradigm known to reveal a sex difference in which male rats performs better than female rats. Therefore, to investigate the sex-related effect of in utero and lactational exposure to TCDD on brain functions, and to elucidate the neurochemical basis of the developmental neurotoxicity of TCDD, we examined the effect of exposure of dams to a low dose of TCDD on contextual fear conditioning of male and female offspring and the conditioning-accompanied CREB activation in the hippocampus.

The experiments were carried out under the guidelines of the Ethical Committee of Animal Experiments of the University of Yamanashi. Adequate measures were taken to minimize pain or discomfort. TCDD (Cambridge Isotope Laboratory, Andover, Massachusetts, USA) was dissolved in nonane, followed by further dilution with corn oil. Timed pregnant Wistar rats (Japan SLC, Shizuoka, Japan) were maintained in a light- (lights on from 06:00 to 18:00 h) and temperature-controlled environment and provided food and tap water ad libitum. On gestation day (GD) 15, TCDD (1 μg/kg body weight) was administered by gavage to pregnant rats. The dose of TCDD was chosen based on the results reported by our previous study and others [9,14]. Pregnant rats given an equivalent volume of corn oil served as controls. The size of litters was standardized on postnatal day 2 to three males and three females. Offspring was gonadectomized at 11 weeks of age to exclude the possible effect of a change in gonadal hormone levels due to neonatal TCDD treatment; and one week later, it was subjected to a contextual fear conditioning test as described previously [13]. Briefly, prior to training, rats received 3-day habituation twice a day. On the day of testing, the rats were placed in a conditioning chamber and allowed to explore for 3 min. A foot shock with an intensity of 0.6 mA was delivered three times at 1, 9, and 18 s after the onset of the tone conditioned stimulus (CS). The rats were then allowed to recover for 30 s in the conditioning chamber and returned to their home cage. One hour later, the rats were again placed in the conditioning chamber in which they had been trained and were tested for a 5-min period during which no tone CS was presented. Conditioning was assessed by measuring the time spent in freezing during the testing period. Freezing behavior was defined as cessation of all but respiratory movement. Freezing time is expressed as the percentage of the time spent in freezing in a total 5-min test period. Immediately after completion of testing, the rats were decapitated, and brains were removed within 90 s and stored at −80 °C until sections were made. Ten-micrometer thick coronal sections were cut using a cryostat with reference to the atlas of Paxinos and Watson [17]. Hippocampal sections located 2.9 mm posterior to the bregma suture were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.0 for 15 min and stored at −20 °C in a cryoprotectant (25% ethylene glycol, 25% glycerin in 0.05 M phosphate buffer) until immunostaining was done. The sections were immunostained for CREB and its phosphorylated form (pCREB). They were treated with 3% H_2O_2 for 10 min and 10% normal horse serum in Tris-buffered saline for 1 h. The sections were then incubated with either anti-pCREB antibody at 1:50 dilution (Cell Signaling Technology, Beverly, Massachusetts, USA), which specifically recognizes CREB phosphorylated at Ser133, or anti-CREB antibody at 1:200 dilution, which detects both the phosphorylated and unphosphorylated forms of CREB, at 4 °C for 48 h. The immunoreactive specificity was validated by the result that no staining of hippocampal tissue was found with omission of the primary antibodies. Tissue sections were incubated with biotinylated anti-rabbit IgG (Vector, Burlingame, California, USA) at 1:100 dilution for 3 h followed by avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit, Vector) for 3 h. The peroxidase reaction was run for 15 min using a DAB Peroxidase Substrate Tablet Set (Sigma, St. Louis, Missouri, USA) in the presence of 1% nickel ammonium sulfate. Microscopic images of randomized sections were captured with a high-sensitivity CCD camera (DP-50, Olympus, Tokyo, Japan) and transferred into a computer. Neurons with nuclei intensely immunostained for pCREB or CREB were judged to be immunoreactive (ir) and counted. Two independent counts were made from at least two different sections per animal and averaged. Taking the possibility of variations in the cell density or cell number in hippocampal sections between groups into consideration [15,18], we chose the ratio of the pCREB-ir cell number counted in a given area of a section to the CREB-ir cell number in the corresponding area of the adjacent section (% pCREB-ir cell number), rather than the absolute cell number, as a measure of CREB phosphorylation, to minimize the variations. The experimental data were analyzed by one-way ANOVA followed by Fisher's PLSD test.

Exposure to TCDD on GD 15 caused a slight but significant decrease in body weight measured at 11 weeks of age as compared to vehicle-treated, control rats (female control versus TCDD-treated, 237.6 ± 5.0 (mean ± SEM) versus 221.7 ± 6.1 g, $n = 9$, $p < 0.05$; male control versus TCDD-

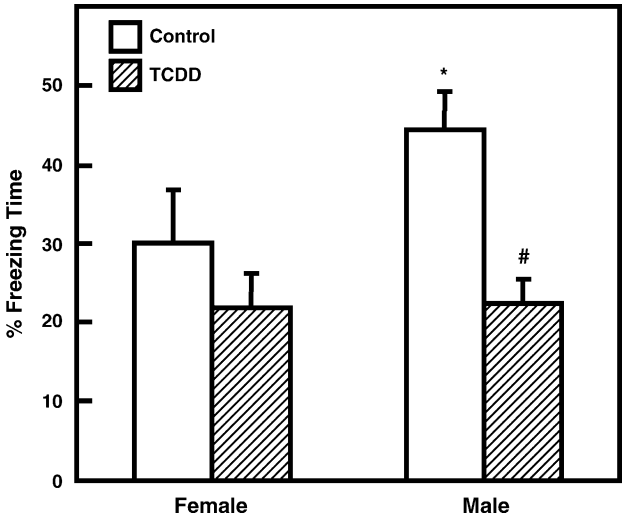

Fig. 1. Effect of perinatal TCDD treatment on freezing behavior after contextual fear conditioning in female and male rats. Rats, exposed to either 1 μg/kg body weight TCDD (hatched column) or vehicle (open column) on gestation day 15, were subjected to contextual fear conditioning at 12 weeks of age. The rats received a foot shock of 0.6 mA and 1 h after training, they were tested for freezing behavior. Freezing is expressed as the percentage of the time spent in freezing in a total 5-min test time. Each bar indicates mean ± S.E.M. of eight or nine animals. *Significantly different from females; #significantly different from controls at $p < 0.05$.